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Reduction of Heat-Shock Protein-70 After Prolonged Treatment With Retinoids: Biological and Clinical Implications

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Heat shock proteins (HSPs) are a group of highly conserved polypeptides involved in cellular response to heat or other physical or chemical stresses. It has been recently reported that HSPs could play a role in cellular differentiation. In this study we have evaluated, by a cytofluorimetric method, the presence of HSP-70 in HL-60 cells during treatment with all-trans retinoic acid (ATRA), 9-cis retinoic acid (9-cis RA), and 13-cis retinoic acid (13-cis RA). After 1 and 3 days of incubation at 10^{-7} M, HSP-70 did not show any variation compared to control; prolonging the exposure, together with the appearance of cellular differentiation along the granulocytic pathway and apoptosis, a progressive decrease of HSP-70 was observed that, after 8 days of treatment, was reduced by 40% with ATRA and by 28% with 9-cis RA compared to untreated samples, while only minimal changes were evident by incubating the cells with 13-cis RA. Reduction of HSP-70 was not associated with decreased protein synthesis, as demonstrated by [^{3}H] leucine incorporation. Double labeling with propidium iodide showed a decrease in HSP-70 in all the phases of the cell cycle concomitant with a reduced percentage of cycling cells in ATRA-treated samples. Dot blot and Northern blot analysis demonstrated no change in HSP-70 mRNA after retinoid treatment, thus suggesting a post-transcriptional regulation of the phenomenon. This reduced production of HSP-70 caused by ATRA and by 9-cis RA, though to a lesser extent, could render the cells more sensitive to cytotoxic agents and could provide the rationale for the efficacy of ATRA + chemotherapy combinations. Am. J. Hematol. 56:143–150, 1997. © 1997 Wiley-Liss, Inc.

Key words: HSP-70; retinoids; ATRA

INTRODUCTION

Heat shock proteins (HSPs) are a group of highly conserved polypeptides [1] that play an essential role in normal cellular physiology, as their synthesis is activated after exposure to high temperature or other physical or chemical stresses [2–4]. HSP-70 represents a family of homologous proteins that are involved, as molecular chaperones, in a variety of intracellular processes. In particular they bind to newly syntesized polypeptides and prevent incorrect folding [5]; moreover they participate in the transportation of proteins to endoplastic reticulum and mitochondria [6] and in the targeting of proteins to lysosomes for degradation [7]. It has been reported that the production of HSP-70 could be modified during cellular differentiation, either in a mouse embryo model [8] or in neoplastic models, such as HL-60 cells induced to differentiate by phorbol 12-myristate 13-acetate (TPA) [9,10], all-trans retinoic acid (ATRA), dimethyl sulfoxide (DMSO) [10], or N-methylformamide [11].

Neoplastic cells show a higher expression of HSP-70 than their normal counterpart [12,13], and an increased level of protein appears to be associated with tumor progression [14]. In this paper we aimed to quantify, by flow cytometric analysis, the variation in the intracellular con-

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tent of HSP-70 in HL-60 cells before and after prolonged treatment with three different retinoid derivatives, ATRA, 9-cis retinoic acid (9-cis RA), and 13-cis retinoic acid (13-cis RA). The clinical activity of retinoids has been demonstrated in several neoplastic models, including skin cancer [15], head and neck cancer [16], lung cancer [17], and in acute promyelocytic leukemia (APL) or in other hematological malignancies [18–20]; thus, it could be interesting to evaluate their effects on HSP-70 in a differentiation sensitive neoplastic system, in order to obtain useful clinical insights.

MATERIALS AND METHODS

Reagents

Monoclonal antibody against both constitutive and heat-inducible forms of HSP-70 was purchased from Sigma Chemicals (St. Louis, MO). ATRA and 9-cis RA were kindly provided by Hoffman-la Roche (Basel, Switzerland); 13-cis RA was purchased from Sigma. Retinoids were first dissolved in absolute ethanol; further dilutions were performed in RPMI 1640 medium (Gibco Europe, Paisley, UK).

Cell Line

Exponentially growing human acute myeloid leukemia cell line HL-60 [21] was used throughout the study. Cells were cultured in 75 mm^2 sterile plastic flasks in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco) and passed 3 times weekly. Flasks were kept in a humidified incubator at 37°C in an atmosphere containing 5% CO_2. Under these conditions, HL-60 doubling time was approximately 24 hr.

Culture With Retinoids

HL-60 cells (2×10^6) were resuspended in 20 ml of RPMI + 10% FCS. ATRA, 9-cis RA, and 13-cis RA were added at 10^{-7}M. Control cultures received the same amount of media, without the drug. Three times weekly, cultures were passed and reseeded with fresh medium and drugs. After 1, 3, 5, and 8 days of incubation, aliquots of cells were counted, assessed for viability by trypan blue dye exclusion, and analyzed as described next. Controls and 8-day retinoids-treated cells were also heat-shocked (20 min at 45°C) and intracellular HSP-70 was subsequently quantified. Each experiment was conducted in triplicate and repeated a minimum of 3 times.

Intracellular HSP-70 Expression

After various retinoid treatments, 2×10^6 HL-60 cells were fixed and permeabilized with paraformaldehyde/triton. After washing with ice-cold phosphate buffered saline (PBS), cells were incubated with 5 μl of a 1:40 dilution of anti HSP-70 monoclonal antibody at room temperature for 15 min. Cells were subsequently washed with PBS and incubated with 5 μl of FITC-labelled goat-anti mouse IgG (DAKO Corporation, Carpinteria, CA). Cells were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with Lysis II software. Data analysis was performed upon acquisition of at least 10,000 events.

Cell Cycle Analysis

After the entire procedure of HSP-70 labelling, the cells were stained with propidium iodide (PI) in PBS at 4°C for 20 min. Cells were subsequently analyzed as described above.

[^{3}H] Leucine Incorporation

After 1, 3, 5, and 8 days of incubation with retinoids, 2×10^6 cells were resuspended in 10 ml of leucine-free RPMI medium supplemented with 10% FCS. After 24 hr of incubation, 5 μl of [^{3}H] leucine (Amersham International, Amersham Place, UK; specific activity 47 Ci/mmol) were added, and incubation was continued for 6 hr more. Cells were subsequently washed twice and the radioactivity in the cell pellets was quantified by liquid scintillation techniques.

RNA Analysis

At baseline and after 8 days of incubation with ATRA, total RNA was extracted from 50×10^6 cells with RNAzol (Bioteck, Galveston, TX) using the manufacturer's procedure. For dot-blot analysis, 20 μg of RNA was spotted onto nylon filters (Amersham) in scalar dilutions. The prehybridization was performed in a solution containing 50% formamide (Sigma) 5 × SSC, 0.02% SDS, 0.1% sarkosil for 2 hr at 42°C. The hybridization was carried out in the same solution containing 100 ng/ml of digoxigenin-labelled HSP-70 c-DNA [22,23] for 10 hr at 42°C. The stringency washes were performed as follows: 2 × SSC 0.1% SDS for 15 min at room temperature, 0.5 × SSC 0.1% SDS for 30 min at 65°C twice and 0.5 × SSC 0.1% SDS for 5 min at room temperature. The detection of hybrids was carried out as previously described [23]. The dot-blot was stripped and rehybridized with XbaI/PstI GAPDH (glyceraldehyde-3-phosphate dehydrogenase) 780 bp digoxigenin labelled probe. The Northern blot was carried out using 20 μg of total RNA electrophoresed in 1.2% agarose-formaldehyde gel and blotted onto a nylon membrane. The hybridization and detection procedure was performed as described before.

Statistical Analysis

Data were analyzed by Student's *t*-test; differences were considered significant when $P < 0.05$ (two-tailed test).

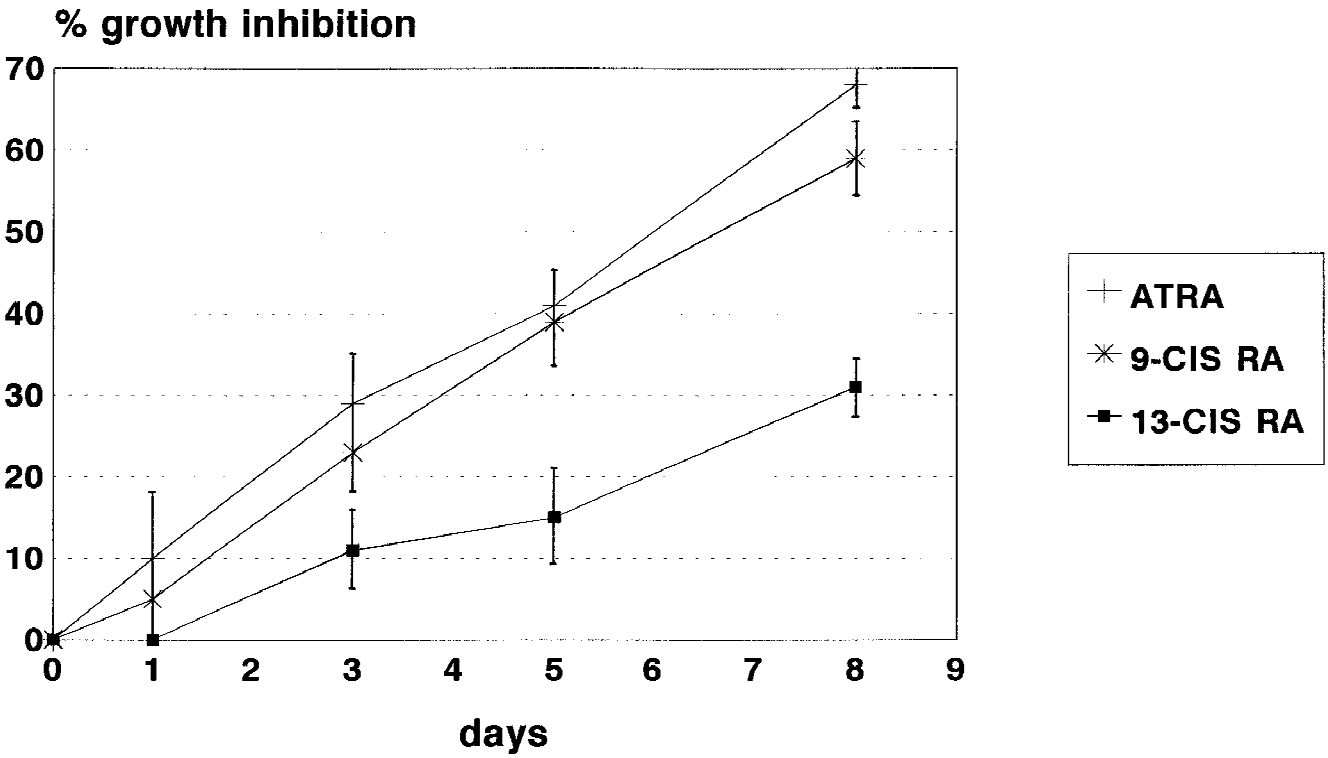

Fig. 1. In vitro activity of retinoids on HL-60 cell growth. Data are expressed as percent of control (no drug) growth inhibition.

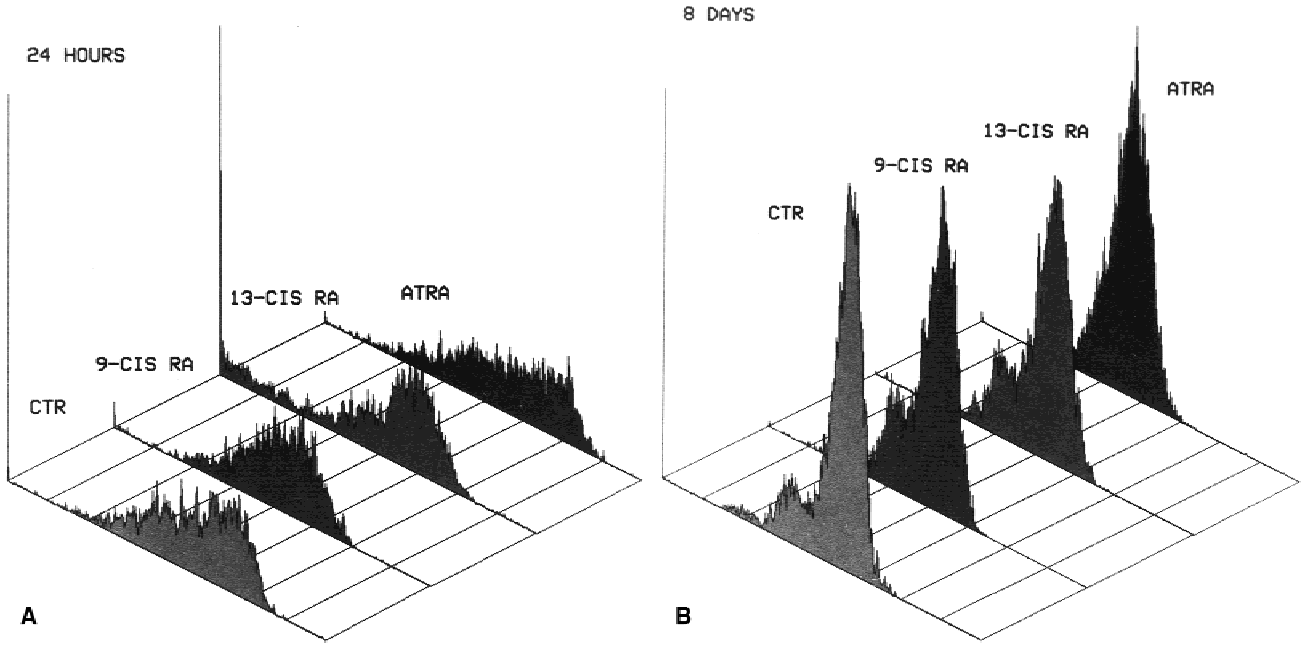

Fig. 2. Representative sample of flow cytometric analysis of intracellular HSP-70 in HL-60 cells after treatment with retinoids. A: 24 hours exposure. B: 8 days exposure. X axis: channel number. Y axis: number of cells. Each experiment was performed in triplicate and repeated a minimum of 3 times. Statistical analysis is reported in Table I.

TABLE I. Cytofluorimetric Analysis of HSP-70 Protein†

	Mean Fluorescence Intensity (sample mean channel:control mean channel ratio)			
	Control	ATRA	9-cis RA	13-cis RA
Baseline	52.28 ± 2.8	51.78 ± 1.7	53.62 ± 3.1	50.74 ± 3.3
24 hr	51.06 ± 3.5	51.61 ± 2.4	55.82 ± 5.3	55.52 ± 5.1
3 days	53.85 ± 5.1	50.47 ± 2.8	51.97 ± 7.2	54.39 ± 3.7
5 days	57.00 ± 4.3[a]	46.35 ± 1.8[a]	49.95 ± 1.4	57.41 ± 2.7
8 days	56.94 ± 4.1[b,c]	33.85 ± 2.5[b]	40.80 ± 3.2[c]	52.32 ± 5.0
8 days + heat	60.98 ± 1.9[d,e]	49.82 ± 4.3[d]	52.81 ± 3.2[e]	55.00 ± 2.9

†Values are expressed as mean ± SD of at least three experiments performed in triplicate.
[a]$P = 0.016$.
[b]$P = 0.001$.
[c]$P = 0.005$.
[d]$P = 0.014$.
[e]$P = 0.019$.

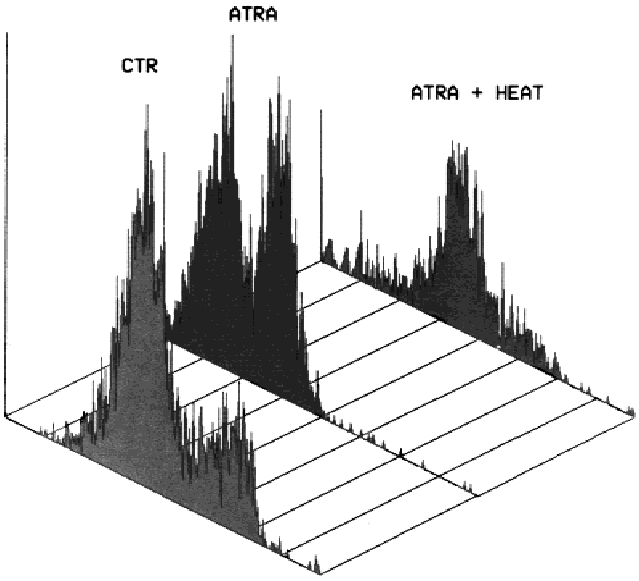

Fig. 3. Evaluation of HSP-70 after 8 days treatment with ATRA and subsequent heat-shock.

RESULTS

ATRA and 9-cis RA caused an exposure-dependent growth inhibition of HL-60 cells, while 13-cis RA was less effective (Fig. 1). Differentiation along the granulocytic pathway was progressively observed, together with the appearance of cells showing chromatin condensation and nuclear fragmentation with Giemsa stain, thus suggesting the occurrence of apoptosis, as previously reported [24]. Cellular mortality, however, did not exceed 20% in samples incubated longer, as assayed by trypan blue dye exclusion.

HSP-70 protein is highly expressed in HL-60 cells, in fact flow cytometric analysis of HSP-70 demonstrated a percentage of HSP-70 positive cell that always exceeded 90%; evaluation of the mean fluorescence intensity (MFI = sample mean channel: control mean channel ratio, where control = staining with isotype-matched FITC) showed a high level of protein expression in untreated cells; after 1 and 3 days no significant variation in the amount of protein was observed in retinoid-treated samples; after 5 and, above all, 8 days of incubation, however (Fig. 2), HSP-70 was significantly decreased in ATRA (40%, $P = 0.001$) and, to a minor extent, in 9-cis RA treated cells (28% decrease, $P = 0.005$) compared to control (Table I). 13-cis RA, instead, appeared to be almost ineffective (8% decrease after 8 days of incubation). Exposure of cells to heat-shock (45°C for 20 min) showed a partial reversion of this phenomenon (Table I and Fig. 3), and probably demonstrated that molecular mechanisms controlling HSP-70 activation remained unchanged. Cell cycle distribution of HSP-70 was also evaluated, by double labeling with PI and anti-HSP-70 antibody. As shown in Figure 4, a high level of HSP-70 was present in all the phases of the cycle in untreated samples. S and G2-M cells, however, displayed a higher fluorescence intensity. This result is concordant with previously reported data [25], according to which HSP-70 is not regulated during the cell cycle, even though it is slightly increased in S-G2-M cells. In Table II are the percentages of HSP-70 positive cells in the different phases of the cell cycle. The differentiating activity of ATRA and 9-cis RA contributed to cause a reduction in the percentage of cells in S and G2-M phases; as these cells are known to possess a higher amount of protein (higher HSP-70 fluorescence intensity, Fig. 4) this could partially explain the reduction in HSP-70 observed after ATRA treatment.

It could be argued that the decrease in intracellular HSP-70 observed after prolonged exposure to ATRA or 9-cis RA was due to a decrease in total protein synthesis,

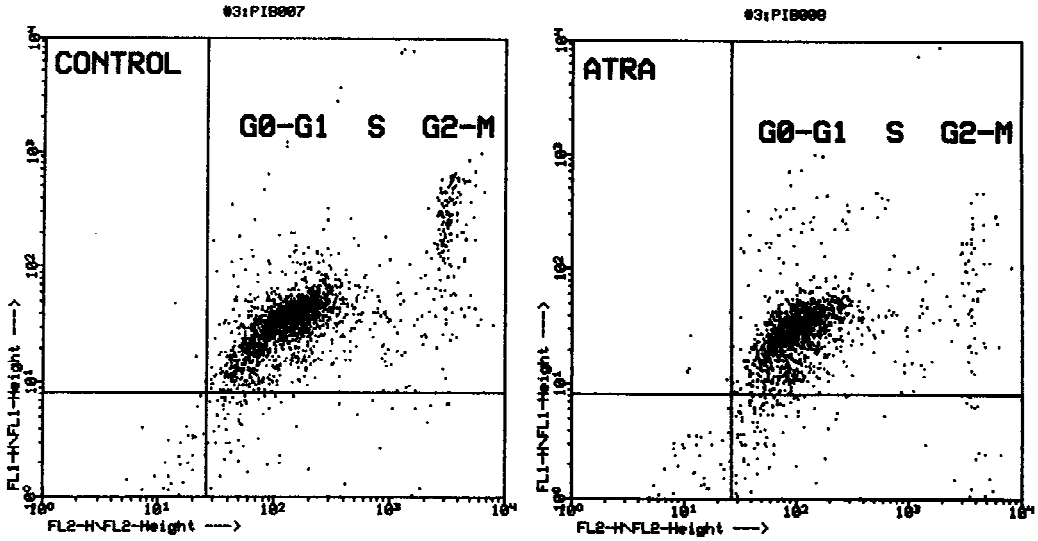

Fig. 4. Example of cell-cycle distribution of HSP-70 in one sample of HL-60 cells after 8 days treatment with ATRA (right): comparison to control (left).
X axis: red fluorescence = cell cycle evaluation by propidium iodide stain. Y axis: green fluorescence = HSP-70 expression by anti HSP-70 monoclonal antibody + FITC-labelled goat-anti mouse IgG.

as a differentiation-related phenomenon. However, this was not the case, as [^{3}H] leucine incorporation remained stable throughout the study (Fig. 5). RNA analysis by dot blot and Northern blot hybridization showed no difference in untreated and ATRA-treated cells (Fig. 6), this result was confirmed by Phosphor Imager analysis (Bio-rad, Hercules, CA). A post-transcriptional regulation of this phenomenon is thus suggested.

DISCUSSION

HSPs play a central role in cellular response to environmental stress [2,4]. It has been recently demonstrated that the intracellular content of some of the members of the HSP family is modified during the course of cellular differentiation. For example, the small HSP-28 has been described to increase during differentiation of K562 cells into erythroblasts promoted by hemin or sodium butyrate [10]. All the same, HSP-28 undergoes a transient increase in HL-60 cells upon treatment with ATRA or with TPA [10,26,27]. The behavior of HSP-70 is more controversial. Induction of differentiation with TPA causes an increase in HSP-70 mRNA in HL-60 cells [9]. These data are confirmed by Mivechi et al. [10] who, however, do not detect any change in HSP-70 mRNA or protein after treatment with ATRA. In this paper we demonstrate a reduction in HSP-70 protein level after exposure of HL-60 cells to differentiation-inducing retinoids, such as ATRA and, though to a minor extent, 9-cis RA. This different finding could be due to a longer exposure, as we were not able to detect any change with short-term treatment. The reduction in intracellular HSP-70 appears to be post-transcriptionally regulated. In fact, mRNA is unchanged, as demonstrated by dot-blot and Northern blot hybridization. A discordant behavior between protein and mRNA synthesis has already been reported [10,26], thus supporting our data. At present, we cannot find an explanation for the decrease in HSP-70 caused by ATRA and 9-cis RA, and the lack of activity shown by 13-cis RA; while the latter could be partially explained by the

TABLE II. Cytofluorimetric Evaluation of HSP-70 Through the Cell Cycle*

	% HSP-70 positive cells							
	G0-G1		S		G2-M		Total	
	% cells	%HSP+	% cells	%HSP+	% cells	%HSP+	% cells	%HSP+
Control	86.11	86.02	1.81	1.75	12.08	12.04	100	99.81
ATRA	92.99	87.79	0.72	0.70	6.29	3.31	100	91.80
9-cis RA	91.65	89.75	1.53	1.06	6.82	6.12	100	96.93
13-cis RA	89.08	87.98	1.27	1.23	9.65	9.52	100	98.73

*Cell cycle was analyzed after 8 days of treatment with retinoids. Cells were double stained with anti HSP-70 monoclonal antibody and FITC-labelled goat anti-mouse IgG and subsequently with propidium iodide for cell cycle evaluation. Results are expressed as the mean of at least three experiments.

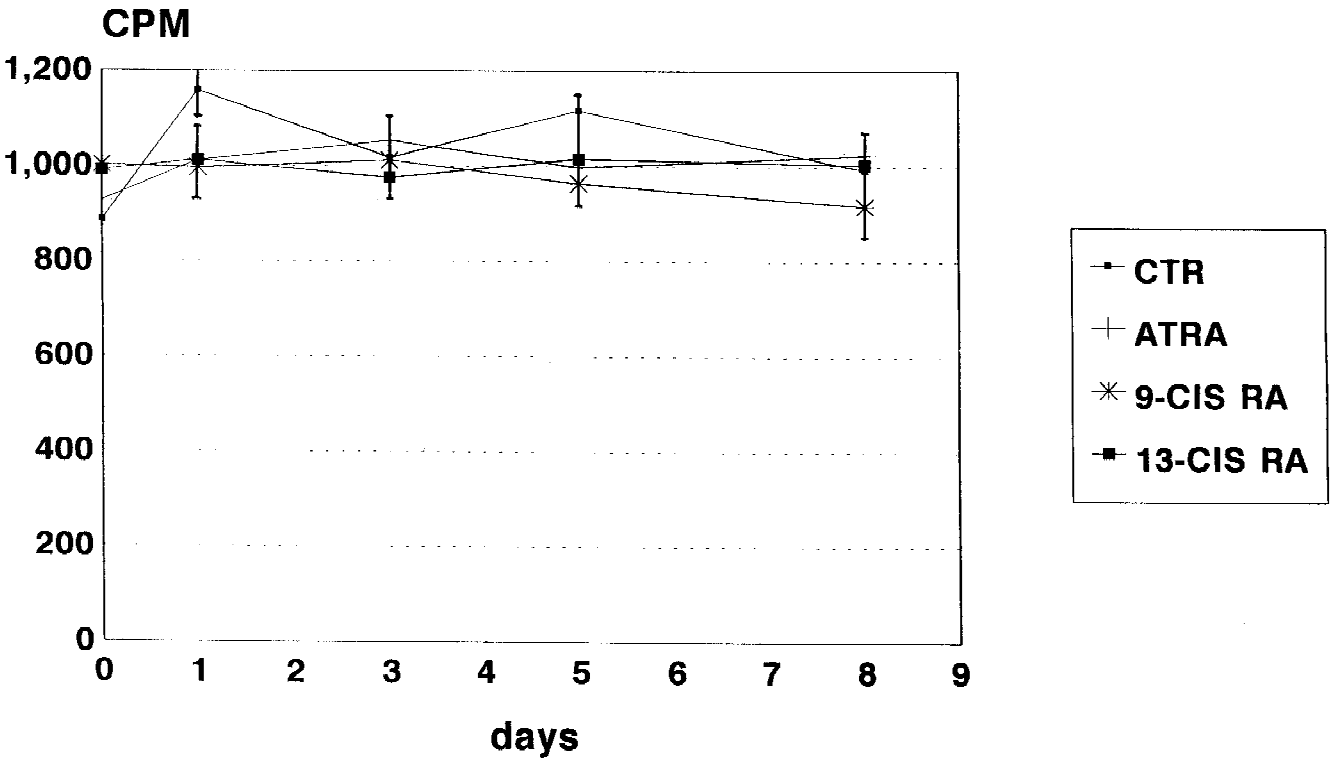

Fig. 5. [3H] leucine incorporation in HL-60 cells throughout 8 days.

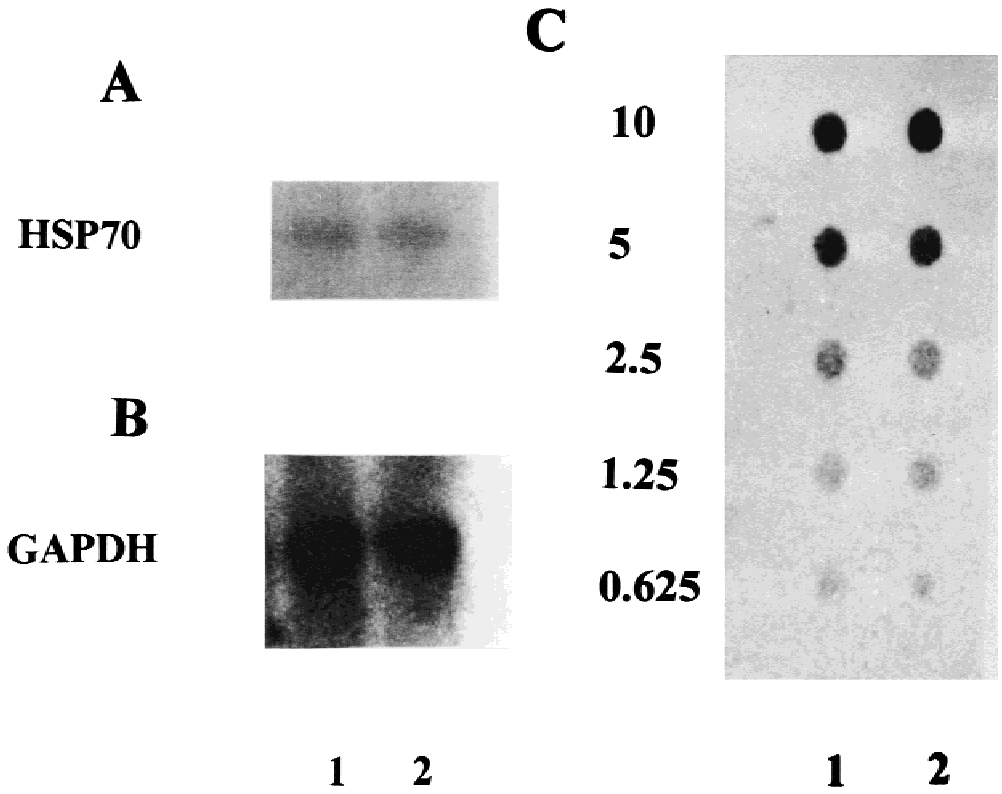

Fig. 6. HSP-70 nRNA analysis. A: Northern blot. Lane 1 = control, Lane 2 = ATRA treated cells (8 days). B: Northern blot of GAPDH mRNA. Lane 1 = control, Lane 2 = ATRA treated cells. C: Dot-blot. Lane 1 = control, Lane 2 = ATRA treated cells. On vertical axis are reported the scalar dilutions (in µgrams) of RNA spotted on the filters.

fact that ATRA and 9-cis RA are more potent inhibitors of growth and inducers of differentiation in HL-60 cells [28] compared to 13-cis RA, the first question deserves further investigation in order to better understand the relationship occurring between retinoid activity and HSP-70. ATRA and 9-cis RA are able to determine apoptosis in HL-60 cells [25,29,30] and it has been recently reported that treatment of cell lines with antisense oligomers against HSP-70 causes inhibition of proliferation and induction of apoptosis [31]; this could suggest a common pathway that leads to programmed cell death. Investigation on oncogenes related to apoptosis, however, has not provided any conclusive evidence so far; in fact mutant p53, which is known to be associated with HSP-70 [32], is not present in HL-60 cells [21], while c-myc, which is largely expressed in HL-60 cells and downregulated during granulocytic differentiation [33], does not appear to be involved in the decrease of HSP-70 caused by N-methylformamide [11]. Whatever the mechanism of reduction of HSP-70 could be, however, these effects of ATRA and 9-cis RA prompt some considerations. Several studies pointed out that tumor cells overexpress HSPs [12,14] and this could represent a proliferative advantage in comparison to normal cellular populations [34], so reduction in HSP-70 could theoretically contribute to reverse this advantage. Furthermore, ATRA is currently used in the treatment of APL and other retinoids are frequently proposed as therapeutic approaches for solid tumors such as skin, head and neck, and lung cancer [15–17]. As a decrease in HSP-70 could render the cells more sensitive to cytotoxic agents [35], our findings could represent a rationale for ATRA + chemotherapy combination schedules.

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